

Cloning of *nod* Gene Regions from Mesquite Rhizobia and Bradyrhizobia and Nucleotide Sequence of the *nodD* Gene from Mesquite Rhizobia

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Nitrogen-fixing symbiosis between bacteria and the tree legume mesquite (*Prosopis glandulosa*) is important for the maintenance of many desert ecosystems. Genes essential for nodulation and for extending the host range to mesquite were isolated from cosmid libraries of *Rhizobium* (mesquite) sp. strain HW17b and *Bradyrhizobium* (mesquite) sp. strain HW10h and were shown to be closely linked. All of the cosmid clones of rhizobia that extended the host range of *Rhizobium* (*Parasponia*) sp. strain NGR234CS to mesquite also supported nodulation of a Sym[−] mesquite strain. The cosmid clones of bradyrhizobia that extended the host range of *Rhizobium* (*Parasponia*) sp. strain NGR234CS to mesquite were only able to confer nodulation ability in the Sym[−] mesquite strain if they also contained a *nodD*-hybridizing region. Subclones containing just the *nodD* genes of either genus did not extend the host range of *Rhizobium* (*Parasponia*) sp. to mesquite, indicating that the *nodD* gene is insufficient for mesquite nodulation. The *nodD* gene region is conserved among mesquite-nodulating rhizobia regardless of the soil depth from which they were collected, indicating descent from a common ancestor. In a tree of distance relationships, the NodD amino acid sequence from mesquite rhizobia clusters with homologs from symbionts that can infect both herbaceous and tree legumes, including *Rhizobium tropici*, *Rhizobium leguminosarum* bv. *phaseoli*, *Rhizobium loti*, and *Bradyrhizobium japonicum*.

The nitrogen-fixing symbiotic relationship between legumes and root nodule-forming bacteria requires gene expression from both partners. Bacterial genes regulating root nodule elicitation and host specificity are conserved among symbionts if they function as common nodulation determinants. Common *nod* genes include early determinants, such as *nodABC*, that are interchangeable among species. Common *nod* genes, such as *nodB* (22) and *nodC* (2, 7), as well as host specificity genes are involved in the synthesis of modified *N*-acetylglucosamine derivatives that act as signals of nodule formation (40). Loci that have roles in host specificity, such as *nodE*, do not complement mutations in symbionts of phylogenetically more distant plant hosts (13). Some *nodD* alleles act as common *nod* loci, while the regulatory response of other *nodD* alleles to plant signals is plant host specific (reviewed in references 14 and 38). Flavonoids and related compounds (32, 34) together with NodD induce *nod* gene activation at promoter regions, termed *nod* boxes, which are found upstream of most nodulation operons. Flavonoids may also act as inhibitors of *nod* gene activation, depending on the specific *nodD* allele (10).

Our work focuses on bacteria that engage in nitrogen-fixing symbiosis with the desert tree legume mesquite (*Prosopis glandulosa*). Deserts are limited in both water and nitrogen. Mesquite's deep-rooting ability to access groundwater and its ability to fix nitrogen symbiotically are adaptive mechanisms that ensure the success of this plant in arid ecosystems. Both rhizobia and bradyrhizobia nodulate mesquite at Harper's Well, Calif., in the Sonoran Desert. Their populations are concentrated in the surface and deep root zones. They reach their

greatest numbers at 4 to 6 m in the phreatic rooting zone, just above groundwater (21, 44). The formation of steep physical and chemical gradients across the Harper's Well soil profile is estimated to have begun up to half a millenium ago after the recession of Lake Cahuilla (18, 43, 44). These longstanding gradients are proposed to constitute strong selection for microbial genetic divergence (20, 41). Phenotypic characterization of mesquite rhizobia and bradyrhizobia isolated from surface, intermediate, and deep phreatic soils, all of which contain nitrogen gas, demonstrated physiologic differences that reflect adaptation to specific conditions related to soil depth, moisture, and nutrient concentrations (45).

Recently, we provided evidence that the Harper's Well (HW) strains identified by Waldon et al. (45) were populations that diverged genetically (41). Evidence from pairwise restriction fragment conservation implicated clonal ancestries for all surface, intermediate, and deep soil isolates of either genus (41). Both genera were shown to have diverged at *ndv*-hybridizing regions.

Most research on the symbiotic process has focused on herbaceous crop legumes and their bacterial partners. In order to gain an understanding of this process in other important legumes, we investigated the association between mesquite and its microsymbionts. Our objectives were to isolate nodulation regions of the two genera by host range extension and functional complementation, to subclone and assess the possible role of *nodD* regions in specifying mesquite as a host, and to analyze the nucleotide sequence of the *nodD* gene of mesquite rhizobia. The *nod* and mesquite host range genes were selected from libraries contained in an alfalfa (*Medicago sativa*) rhizobium, a *Parasponia* rhizobium, or a Nod[−] mesquite rhizobium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown at 37°C on Luria-Bertani medium (37). For electroporation, growth of

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid(s)	Phenotype and/or genotype	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ Φ 80 α <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 hsdR17 deoR supE44 thi-1 recA1 gyrA96 relA1</i>	Laboratory stock (37)
<i>Rhizobium</i> (<i>Parasponia</i>) sp. strain NGR234	Broad-host-range symbiont but Nod ⁻ on mesquite	B. Rolfe (30)
<i>Rhizobium</i> (<i>Parasponia</i>) sp. strain NGR234CS	Cm ^r Sm ^r derivative of NGR234	This study
<i>Rhizobium</i> sp. strain HW27c	Harper's Well collection, Sym ⁻ Nod ⁻	20, 41
<i>Rhizobium</i> sp. strain HW27cCS	Cm ^r Sm ^r derivative of HW27c	This study
<i>Rhizobium</i> (mesquite) sp. strain HW17b	Harper's Well collection	20, 41
<i>Bradyrhizobium</i> (mesquite) sp. strain HW10h	Harper's Well collection	20, 41
Plasmids		
pLAFR2	Tc ^r IncPI cosmid	M. Silverman (12)
pRK2013	Ap ^r ColE1 <i>ori</i>	G. Ditta (9)
pBluescriptI/KS	Ap ^r	Stratagene
p27A1, p27D2	<i>nod</i> region and mesquite-host-range gene(s) in pLAFR2, from HW17b cosmid library in <i>Rhizobium</i> sp. strain HW27cCS	This study
p27A3, p27B1	<i>mel/nod</i> region and mesquite-host-range gene(s) in pLAFR2, from HW17b cosmid library in <i>Rhizobium</i> sp. strain HW27cCS	This study
p234G1, p234H1	<i>mel/nod</i> region and mesquite-host-range gene(s) in pLAFR2, from HW17b cosmid library in <i>Rhizobium</i> sp. strain NGR234CS	This study
pPMT15	p27B1 <i>nodD</i> -containing 7.8-kb <i>Eco</i> RI fragment in pLAFR2	This study
pPMT8	p27B1 <i>nodD</i> -containing 2.7-kb <i>Xho</i> I fragment in pBluescriptI/KS	This study
pPMT9	p27B1 <i>nodD</i> -containing 2.7-kb <i>Xho</i> I fragment in pBluescriptI/KS in orientation opposite to that in pPMT8	This study
pKFG65, pKFG71, pKFG83	<i>nod</i> region in pLAFR2 from HW10h library in <i>Rhizobium</i> sp. strain NGR234CS	This study
pKFG65.2	pKFG65 <i>nodD</i> -containing 4.2-kb <i>Bam</i> HI fragment cloned into pLAFR2	This study

strains after voltage discharge was in SOC broth (37). Growth of strains for transfection of cosmid libraries was in tryptone broth supplemented with 0.2% maltose and 10 mM MgSO₄ (Gigapack protocol; Stratagene). Isolation of cosmid DNA was performed after growth of *E. coli* hosts in Superbroth (35 g of Bacto-tryptone [Difco], 20 g of yeast extract, and 5 g of sodium chloride per liter [adjusted to pH 7.5]) supplemented with the appropriate antibiotics. Symbiotic bacteria were grown as described previously (41). Assays for production of UV-fluorescent Calcofluor-binding exopolysaccharide were performed as described previously (26). Antibiotic concentrations were as follows: tetracycline, 10 μ g/ml; chloramphenicol, 100 μ g/ml; ampicillin, 100 μ g/ml; rifampin, 200 μ g/ml; and streptomycin, 500 μ g/ml. Nutrients, medium components, and other chemicals were purchased from VWR Scientific and Sigma Chemical Co. Enzymes were from New England Biolabs, Stratagene, or Promega.

DNA isolation, electroporation, and conjugation. Isolation of plasmids from *E. coli* was performed as described previously (47). Cosmid DNA was isolated from rhizobial strains by a boiling miniprep technique (23). Single-stranded phagemid DNA was isolated according to instructions provided by U.S. Biochemical Corp. Double-stranded DNA for automated sequencing was isolated by polyethylene glycol precipitation according to instructions provided by Applied Biosystems. Cells were grown overnight at 37°C in Terrific broth (100 ml of sterile 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ added to 900 ml of sterile base broth [12 g of Bacto-tryptone, 24 g of Bacto-yeast extract, 4 ml of glycerol in 900 ml of deionized H₂O]). Each culture (4.5 ml) was centrifuged, and the cells were resuspended in 200 μ l of GTE solution (50 mM glucose, 25 mM Tris [pH 8.0], 10 mM EDTA [pH 8.0]). The suspension was mixed with 300 μ l of 0.2 N NaOH–1% sodium dodecyl sulfate and incubated on ice for 5 min. The lysate was neutralized with 300 μ l of 3.0 M potassium acetate (pH 4.8) prior to incubation on ice for 5 min. After centrifugation, 20 μ g of RNase A per ml was added to the supernatant. After incubation for 20 min at 37°C, the mixture was extracted three times with chloroform. The aqueous phase was combined with an equal volume of 100% isopropanol and centrifuged. DNA pellets were washed with 70% ethanol and vacuum dried. The dried DNA pellet was dissolved in 40 μ l of 0.8 M NaCl, mixed with an equal volume of sterile 13% polyethylene glycol 8000, and placed on ice for 20 min. After centrifugation, the precipitated DNA was washed with 70% ethanol and vacuum dried. Dry pellets were dissolved in 20 μ l of deionized water and frozen until used in sequencing reactions.

Cosmid transfers between *E. coli* and microsymbionts used the triparental mating protocol (9) as modified here. Recipient cells were washed once with M9 salts (27) to remove exopolysaccharide produced by some strains. This step greatly increased the frequency of genetic transfer. After the recipient cells were mixed with *E. coli* strains containing the mobilizing plasmid pRK2013 and cos-

mids, the mixture was filtered onto a membrane and incubated overnight at 30°C. Mating mixtures were resuspended and plated on medium containing the appropriate antibiotics.

Genomic library construction. Genomic fragments between 30 and 50 kb in size were isolated after partial digestion with *Sau*3A and fractionation in 40% sucrose gradients as described previously (37). Libraries were constructed by ligation of fractionated *Sau*3A-digested genomic DNA with *Bam*HI-digested and alkaline phosphatase-treated pLAFR2 (a pLAFR1 derivative with a multiple cloning site inserted at the *Eco*RI site [12]). Overnight ligation was followed by packaging of ligated DNA in λ phage heads with the Gigapack kit (Stratagene). Packaged libraries were transduced into *E. coli* DH5 α , amplified by growth in broth containing 10 μ g of tetracycline per ml, and stored in glycerol stocks at –70°C. Cosmid libraries in *E. coli* DH5 α were transferred into antibiotic-resistant symbionts by triparental mating as described above. Cosmid libraries of the two mesquite symbionts, consisting of ~2,000 pooled colonies from antibiotic-containing plates, were stored in 8.8% dimethyl sulfoxide at –70°C. The libraries appeared to be complete in that there were at least two melanin-producing colonies in each ~2,000-member library.

Plant assays. Mesquite seedlings were inoculated with 10⁸ cells carrying cosmid libraries as described previously (41). Bacteria from mesquite nodules were obtained after streaking of surface-sterilized, squashed nodules on antibiotic-containing media as described previously (42). Bacterial isolates were stored in 8.8% dimethyl sulfoxide at –70°C.

Southern hybridization conditions. Southern hybridization was performed as described previously (41). DNA was digested with restriction enzymes (Stratagene or Promega), and the fragments were separated on 0.8% agarose gels and transferred to GeneScreen Plus (New England Nuclear). The *Rhizobium meliloti* 1021 *nodD1* probe was amplified by PCR (GeneAmp PCR kit [Perkin-Elmer]) with the 8.7-kb *Eco*RI fragment of pRmSL26 (29) as a template and primers 5'-TTGCTCGACGGAGGGCTTCA-3' and 5'-GGATCGCCGTTTCAGGATCA-3'. The *Rhizobium* (mesquite) sp. HW17b *nodD1* probe was the 2.7-kb *Xho*I fragment of pPMT15. Probes were labelled by the random primer method with [α -³²P]dCTP (3,000 Ci/mmol) and the Promega Prime-a-Gene kit. Primers were synthesized in the Microchemical Core Facility at San Diego State University. Restriction enzyme fragments were isolated from agarose by a modification of the "freeze squeeze" technique (27).

Nucleotide sequencing. Nested deletions of the *nodD*-containing 2.7-kb *Xho*I fragment cloned into pBluescriptI/KS in either orientation (pPMT8 and pPMT9) were prepared according to the protocol of Henikoff (16). Deletion subclones were identified after random screening of plasmid DNA or by size selection. DNA from pools of 25 to 50 *E. coli* colonies containing deletions in these

plasmids was separated by electrophoresis in low-melting-point agarose. Slices corresponding to the desired sizes were cut from the gel. The electroconductivity of the agarose slices was reduced by incubation in cold, sterile H₂O for 10 min; the slices were then melted at 68°C for 10 min and held at 45°C until used in electroporation experiments. The primer for sequencing these deletions was a four-base extension of the 3' end of the SK primer (Stratagene). Primers for the complementary strand were 18-mers with 5' ends beginning at base positions 96, 484, and 800, respectively; numbering is according to the sequence published in this work (see Fig. 7). Sequencing was carried out either manually according to protocols provided with Sequenase (U.S. Biochemical Corp. [37]) or by automated DNA sequencing in the Microchemical Core Facility at San Diego State University. The entire gene and its control region were sequenced at least twice in both directions, and the sequence was confirmed with overlapping clones. Alignments and other analyses were carried out through the package of programs configured in DNASYSTEM (39). Sequence similarities were determined with the BLAST program (1) in searches of the combined, nonredundant NCBI database (April 1994).

Nucleotide sequence accession number. The GenBank accession number of the sequence of the *nodD* gene of mesquite rhizobia is L04660.

RESULTS

Construction of genomic libraries of mesquite rhizobia and bradyrhizobia. In order to isolate the genetic information involved in host range extension and nodule formation, genomic libraries of *Rhizobium* (mesquite) sp. strain HW17b and *Bradyrhizobium* (mesquite) sp. strain HW10h were constructed. These strains are from the Harper's Well collection of mesquite-nodulating bacteria (41). Our selection strategy used two antibiotic-resistant derivatives of strains that naturally or because of mutation are unable to nodulate mesquite.

A cosmid clone library of *Rhizobium* (mesquite) sp. strain HW17b genomic DNA was transferred by triparental mating (9) from *E. coli* DH5 α into the broad-host-range *Rhizobium* (*Parasponia*) sp. strain NGR234CS (30) or mesquite *Rhizobium* sp. strain HW27cCS, a Nod⁻ strain (41). A genomic DNA library of *Bradyrhizobium* (mesquite) sp. strain HW10h was similarly established in *Rhizobium* (*Parasponia*) sp. strain NGR234CS. Pools of these new libraries were inoculated onto mesquite seedlings. After several weeks, plants were examined for root nodules, and bacteria were isolated from surface-sterilized root nodules.

Characterization of *nod* clones of mesquite rhizobia. Mesquite plants inoculated with the libraries of mesquite rhizobia contained nodules (7 to 16 nodules per plant) that were removed, and plasmid DNA was isolated from bacteria recovered from the nodules. The average size of the plasmids produced by the two libraries was approximately 50 kb.

From the complementation experiment to select nodulating strains, six plasmids were isolated which enabled either *Rhizobium* (*Parasponia*) sp. strain NGR234CS(p234G1, p234H1) or *Rhizobium* sp. strain HW27cCS(p27A1, p27A3, p27B1, p27D2) to nodulate mesquite. Four of these plasmids also conferred the ability to produce melanin, whereas the other two (p27A1 and p27D2) did not, indicating that the melanin gene is linked to the host specificity determinant and the *nod* region. With the exception of p27A3 digested with *Xho*I, all plasmids contained two *nodD*-hybridizing fragments in common, a 2.7-kb *Xho*I fragment and an 8-kb *Kpn*I fragment (Fig. 1). The plasmid p27A3 carried the hybridizing region linked to the vector fragment.

Plasmids p27A1, p27B1, and p234H1 were moved by conjugation into both *Rhizobium* sp. strain HW27cCS and *Rhizobium* (*Parasponia*) sp. strain NGR234CS. The six transconjugants elicited nodule formation on mesquite, demonstrating that genes extending the host range of *Rhizobium* (*Parasponia*) sp. strain NGR234CS are closely linked to *nod* genes. We focused on one of these three plasmids, p27B1.

Chromosomal DNA from *Rhizobium* sp. strain HW17b and p27B1 DNA were digested with several enzymes singly or in

combination with *Eco*RI, which frees the polylinker entirely from pLAFR2, in order to assess whether *Rhizobium* sp. strain HW17b, like some species, has more than one *nodD* region and to estimate the number of *nodD* genes isolated in p27B1. In each lane, we observed a single band containing singly (lanes a, d, j, l, o, and r) or doubly (lanes b, e, m, p, and s) digested p27B1 (Fig. 2), suggesting that only one *nodD*-hybridizing region was cloned in p27B1. The exception evident in this blot, p27B1 digested with *Kpn*I, is due to star activity (lane g [compare with *Kpn*I lanes in Fig. 1]). Lanes containing *Rhizobium* sp. strain HW17b genomic DNA showed up to four *nodD*-hybridizing fragments (lanes c, k, and t), only one of which is shared with a fragment in p27B1.

The *nodD1* gene of p27B1 is present in genetically divergent HW strains of mesquite rhizobia. We previously provided evidence for the clonal ancestry and genetic divergence (41) of physiologically distinct groups isolated from surface (0.0 to 0.6 m; *Rhizobium* sp. strains HW1b, HW1e, HW10a, HW10d, HW10i, HW27c, HW27a, and HW27e), intermediate (0.6 to 4.0 m; *Rhizobium* sp. strains HW17b, HW17c, HW22a, HW22b, and HW22k), and deep, phreatic (4.0 to 6.0 m; *Rhizobium* sp. strain HW8d) soil depths at Harper's Well. A blot of *Pst*I-digested genomic DNA from these strains was probed with the 2.7-kb *Xho*I fragment containing the *nodD* region of p27B1 to test our expectation of conserved *nod* regions among these isolates. Among the symbionts of mesquite rhizobia, all but two strains have the 6.6-kb *Pst*I fragment containing the *nodD1* gene of p27B1 (Fig. 2, lane 1, and Fig. 3). The exceptions, *Rhizobium* sp. strains HW10a and HW8d (Fig. 3, lanes c and d), contain several hybridizing bands. In addition, a 2-kb hybridizing fragment was observed in all strains.

These results also verify the lack of *nod* hybridization (41) for three HW strains (Fig. 3, lanes a, f, and h). These three strains, including *Rhizobium* sp. strain HW27c, the recipient strain used in this work to select *nod* function, were Nod⁻ on mesquite and did not hybridize with *Klebsiella pneumoniae* *nifHDK* or *R. meliloti* *nodDABC*. Consequently, the symbiotic plasmids of these strains were either absent or contained deletions.

Characterization of *nod* clones of mesquite bradyrhizobia. Cosmid-containing bacteria were isolated from nodules produced on mesquite plants inoculated with the library of *Bradyrhizobium* (mesquite) sp. strain HW10h DNA in *Rhizobium* (*Parasponia*) sp. strain NGR234CS (an average of four nodules per plant). Three plasmids, pKFG65, pKFG71, and pKFG83, were isolated and examined for hybridization to the *R. meliloti* *nodD1* probe. The results shown in Fig. 4 demonstrate that *Bam*HI and *Pst*I digestion of pKFG65 and pKFG71 produced similar hybridizing fragments. The third plasmid, pKFG83, did not hybridize to the *nodD1* probe. When the three plasmids in *Rhizobium* (*Parasponia*) sp. strain NGR234CS were reinoculated onto mesquite seedlings, nodules were produced. Plasmid pKFG83, however, did not restore nodulation ability to *Rhizobium* sp. strain HW27cCS (Sym⁻), while the *nodD*-containing clones pKFG65 and pKFG71 did restore nodulation ability. Common restriction fragments of similar sizes among the three plasmids were evident after single and double digestion with several enzymes (data not shown). These results indicate that the genomic region of bradyrhizobia responsible for extension of the host range of *Rhizobium* (*Parasponia*) sp. strain NGR234CS to mesquite is not *nodD* itself but is closely linked to *nodD*.

Results from probing with the 4.2-kb *Bam*HI *nodD*-containing fragment from *Bradyrhizobium* (mesquite) sp. strain HW10h to a Southern transfer of *Bam*HI digests of *Bradyrhizobium* sp. strain HW10h chromosomal DNA, pKFG65, and

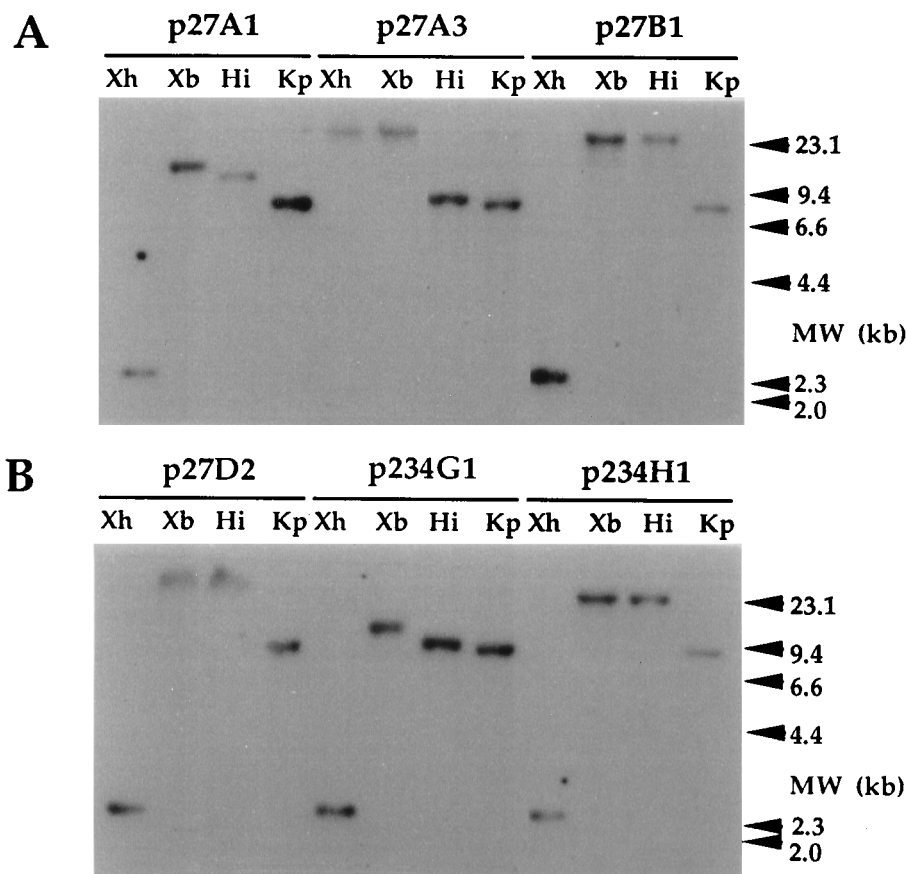


FIG. 1. Hybridization of the *R. meliloti nodD1* probe to *Rhizobium* sp. strain HW17b *Nod*⁺ cosmid DNA. Cosmids obtained from mesquite nodules formed by *Rhizobium* sp. strain HW27cCS carrying p27A1, p27A3, and p27B1 (A) and p27D2 and *Rhizobium* sp. strain NGR234CS carrying p234G1 and p234H1 (B) were digested separately with four enzymes, and prepared blots were hybridized to a radiolabelled, PCR-amplified *R. meliloti nodD1* fragment. Enzymes: Xh, *Xho*I; Xb, *Xba*I; Hi, *Hind*III; Kp, *Kpn*I. MW, molecular size.

pKFG71 are shown in Fig. 5. The single band evident in all three digests suggests that only one copy of the *nodD*-like gene is present in the two plasmids and in the *Bradyrhizobium* sp. strain HW10h genome. An identical pattern was observed when the *R. meliloti nodD1* probe was hybridized to the same blot (data not shown), supporting the inference that only one *nodD* fragment was present. It is possible that more than one genomic DNA fragment comigrated with the cloned fragment, but only one copy exists on the isolated fragment, because a single 0.6-kb *Pst*I fragment was shown to hybridize to the *R. meliloti* probe (Fig. 4).

***nodD1* genes of mesquite rhizobia and bradyrhizobia are insufficient to extend host range.** The *nodD*-hybridizing 7.8-kb *Eco*RI fragment of p27B1 and the 4.2-kb *Bam*HI fragment of pKFG65 (Fig. 6) were subcloned to assess their sufficiency to extend the host range of *Rhizobium (Parasponia)* sp. strain NGR234CS to include mesquite infection. The resulting plasmids, pPMT15 and pKFG65.2, respectively, were mated into *Rhizobium (Parasponia)* sp. strain NGR234CS. Transconjugants carrying the *nodD* regions did not nodulate mesquite. Because of this result and because pKFG83, which does not contain a *nodD* gene, is able to extend the host range of *Rhizobium (Parasponia)* sp. strain NGR234CS, we conclude that *nodD* is insufficient or is not required for mesquite host specificity.

Analysis of the sequence of the *nodD* gene of rhizobia. The 2.7-kb *Xho*I fragment of pPMT15 that hybridizes to *nodD* was subcloned, and the nucleotide sequence of the *nodD* region

was determined (Fig. 7). BLASTN analysis with bases 104 to 153 revealed sequence identities in *nodD* or *nodA* proximal regions of other species that show 70 to 87% correspondence to the *nod* box consensus (46).

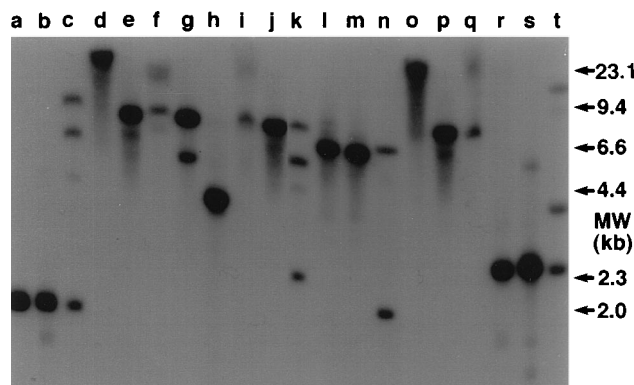


FIG. 2. Hybridization of the *R. meliloti nodD1* probe to restriction enzyme-digested p27B1 and *Rhizobium* sp. strain HW17b genomic DNA. Plasmid p27B1 and *Rhizobium* sp. strain HW17b genomic DNA were singly digested with *Bam*HI (lanes a and c), *Hind*III (lanes d and f), *Kpn*I (lanes g and i), *Eco*RI (lanes j and k), *Pst*I (lanes l and n), *Xba*I (lanes o and q), and *Xho*I (lanes r and t). p27B1 was digested with *Eco*RI and *Bam*HI (lane b), *Eco*RI and *Hind*III (lane e), *Eco*RI and *Kpn*I (lane h), *Eco*RI and *Pst*I (lane m), *Eco*RI and *Xba*I (lane p), and *Eco*RI and *Xho*I (lane s). MW, molecular size.

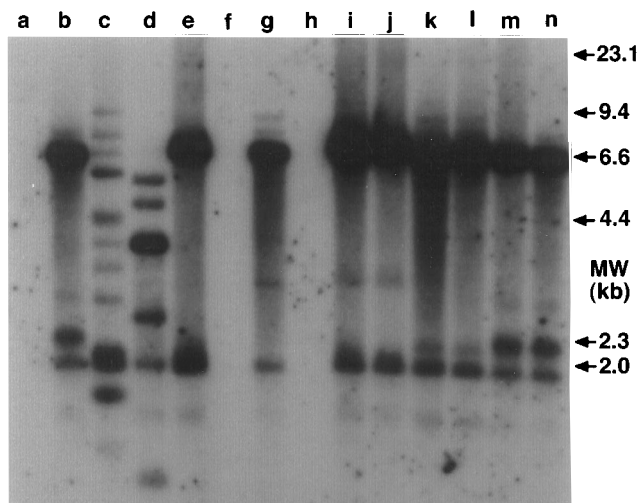


FIG. 3. Hybridization of radiolabelled HW17b *nodD1*-containing 2.7-kb *XhoI* fragment to *PstI* digests of DNA from different HW strains of rhizobia. Lanes: a, HW1b; b, HW1e; c, HW10a; d, HW8d; e, HW10d; f, HW10i; g, HW17b; h, HW27c; i, HW17c; j, HW22a; k, HW22b; l, HW22k; m, HW27a; n, HW27e. MW, molecular size.

BLASTP (1) searches of the protein sequence database of GenBank identified the NodD proteins most similar to *Rhizobium* sp. strain HW17b NodD1 to be those in the multiple alignment shown in Fig. 8. The alignment consists of sequences identified in that search having 88 to 93% amino acid similarity to *Rhizobium* sp. strain HW17b NodD and having corresponding nucleic acid sequence identities of 72 to 80%. The greatest similarity is in the N terminus, which includes the DNA-binding helix-turn-helix (HTH) motif (residues 23 to 42). Seven of the N-terminal 48 residues are substituted once or twice.

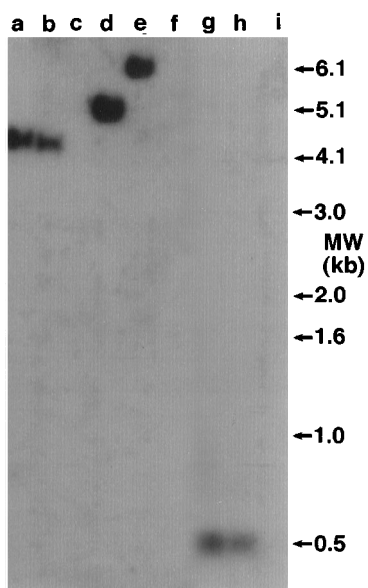


FIG. 4. Hybridization of the *R. meliloti* *nodD1* probe to restriction enzyme-digested *Bradyrhizobium* sp. strain HW10h *nod* region clones. Clones obtained from mesquite nodules formed by *Rhizobium* sp. strain NGR234CS carrying pKFG65, pKFG71, and pKFG83 were digested with *Bam*HI (lanes a, b, and c), *Bgl*II (lanes d, e, and f), and *Pst*I (lanes g, h, and i). MW, molecular size.

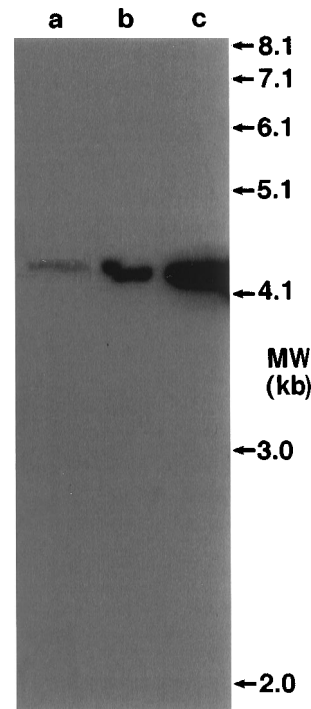


FIG. 5. Hybridization of radiolabelled HW10h *nodD1* region containing 4.2-kb *Bam*HI fragment to *Bam*HI-digested cosmid and HW10h genomic DNA. Lanes: a, HW10h genomic DNA; b, pKFG65 cosmid DNA; c, pKFG71 cosmid DNA. MW, molecular size.

Stretches of full conservation lie in the first 12 residues and the second half of the HTH motif comprising the loop and the recognition helix (4). The proteins are less similar between the end of the HTH motif and the C terminus. Positions 69 to 116 include 47 residues, 15 of which show one or two substitutions. Finally, residues 206 to 261, but most pronounced in the stretch between residues 206 and 232, are relatively conserved. Amino acid substitutions occur at 23 of the 55 positions in this region.

The five NodD sequences of Fig. 8 and the sequence of *Bradyrhizobium* (*Parasponia*) sp. strain NodD form an independent cluster in a tree illustrating distances among selected NodD sequences (bracketed branch in Fig. 9). The strains in this cluster nodulate both herbaceous and tree legumes.

DISCUSSION

Mesquite is a mimosoid tree legume that in desert climates, is faced with a variety of physiological challenges, including little availability of water, low soil nutrient status, and high

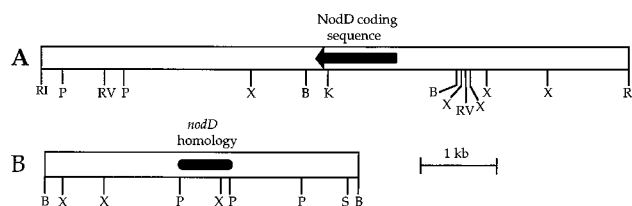


FIG. 6. Restriction endonuclease maps of *nodD* region fragments of mesquite rhizobia (A) and bradyrhizobia (B). Enzymes: B, *Bam*HI; K, *Kpn*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sca*I; X, *Xho*I.

1 ATGTACAGGCTGGTCCACCCCTTCGGTCCCTCTTGGCCACTCGTAAATGCGTCGAAT
 61 GCCCTGATTTCTCGATGATACCTTTCTAATGCAGTAAGCCGTTAAATCCGCAAAATCGAT
 M R F K G L D L
 121 TGTTCGGATGGTACGCATCCGCGTCATGGATGAAAAATGCGTTCAAGGCTCTTGATCTA
 N L L V A L D A L M A E R N L T A A A R
 181 AATCTCTCGTCGCGCTCGACGCCCTGATGGCTGAGCGTAACCTCACGGCGCGGCACGC
 S I N L S Q P A M S A A V A R L R T Y F
 241 AGCATCAACCTCAGTCAGCGCGCATGAGCGCGCGCTCGCTTACGCACCTATTTC
 D D E L F T M I G R E L I P T P R A E R
 301 GATGATGAGCTCTTACGATGATTGGCGCGAACTGATCCGACACCGCTCGCGAACGG
 L A P A V R E T L L H V Q L S I I A G E
 361 CTCGCCCCCGCGGTGCGAGACCCCTGCTCCAGCTCAGCTCTCCATTATCGTGGGAA
 P F N P A Q S D R R F R I I L S D Y A T
 421 CCGTTTAAACCGGCTCAGTCGATCGTCGCTCCGGAATCATCTTCCGATACGCCACA
 L V F F E K I V E R A S R E A P A V S F
 481 CTCGCTCTTTTCGAAAAGATCGTGAGCGTGGCTCGCGGGAAGCTCCCGCCCTGCTTC
 E F L P L A D D Y G D L L R R G D I D L
 541 GAGTTTCTGCTCTTGGCGACGACTATGGGATCTTCTCCGCGCGCGGACATCGATCTT
 L I L P D M F M S D S H P Q A K L F D E
 601 TTGATTCGCGGACATGTTATCGTCAGACGATCATCCCGAGCGAACTATTCGACGAG
 V H V C I G C R S N K Q L S D P L T F E
 661 GTACACGTGTGCATCGGTTGTCGCTCGAAACAGCAACTGTGACACCGCTTACATTCGAG
 S Y M S M G H V A V K F G N T R Q P S I
 721 AGCTACATGTCGATGGGCGATGTCGCGTCAAGTTCCGGAATACCCGCCAGCCCTCGATC
 E E W Y L R E H G L K R R I E V V V Q G
 781 GAGGAATGGTATTTCGCTGAGCAGCGCTCAAGAGACGATCGAGGTTGTCGACAGCGG
 F S M I A P M V S G T E R I G T M P L R
 841 TTCAGCATGATTCGCGCCCATGTTATCGGTCACGAGCGTATAGGACCATGCTCTACGA
 L A Q H F A K A I P L R I V E L P L P L
 901 CTGGCGCAGCATTCGCAAAAGCATACCCCTGCGGATCGTCGAGCTCCCGCTCGCGCTT
 P P F T E A V Q W P A L H N N D P A S L
 961 CCCCCATTACCGAGGCGCTCAATGGCTGCCCTTCAATAATGATCGGCAAGCCTC
 W M R E M L V Q E A S R M G L R R E F T
 1021 TGGATGCGCGAGATGTTAGTACAGGAGCGCTCCGCGATGGGTTTGGCGCGTGAATTACCC
 *

1081 TGAGGTGTCAGCCGCTCTGG

FIG. 7. Nucleotide and deduced amino acid sequences of *Rhizobium* sp. strain HW17b *nodD1* region. The coding strand is shown with the predicted 308-residue protein indicated below it and the termination codon represented by an asterisk. Sequence similarity to the *nod* box consensus reported by Winsor (46) is shown in white letters. Only 5 of 47 bases between the consensus *nod* box and this sequence are different. Amino acid sequence similar to the HTH motif of the LysR family of DNA-binding regulatory proteins is in boldface.

temperatures (31, 44, 45). The deep-rooting strategy of mesquite and its coevolution with rhizobia and bradyrhizobia to symbiotically fix atmospheric nitrogen allow the plant to overcome such adverse conditions. These traits make this widely distributed legume an important contributor to desert ecosystem productivity and nitrogen cycling (36). Although the ecological role of mesquite in arid systems has been studied, we know little of the regulation of symbioses of rhizobia and bradyrhizobia with this woody host. We show here that the bacterial genes involved in mesquite host range determination are linked to *nod* genes. Cosmids of both mesquite rhizobia and bradyrhizobia that are able to restore mesquite-nodulating ability to *Rhizobium* sp. strain HW27cCS (Sym⁻) also extend the host range of *Rhizobium* (*Parasponia*) sp. strain NGR234CS.

A library of *Bradyrhizobium* (mesquite) sp. strain HW10h in *Bradyrhizobium japonicum* I110ARS (25) failed to produce nodules on mesquite plants (data not shown), while a library of *Rhizobium* (mesquite) sp. strain HW17b in *R. meliloti* AK631 (33) produced nodules. Bacteria removed from these nodules were not further characterized, except to examine whether they produced the calcofluor-binding exopolysaccharide

1
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 MRFKGLDLNL LValDALMte RNLTAARSI NLSQPAMSA VaRLrtyF-D
 51
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 ELFTM-GREL VpTPRAe-La paVRealLhI QLSIIswEPF nPaQSDRrFR
 101
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 IILSDYatLV FfEKVV-R-a rEAPaVSFB- lPladdydEl LRRGD-DFLI
 151
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 LPB-FMs--H P-aaLFEE-- VCVGC--Nk- Ls-plTFERy mSMgHVavkf
 201
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 Gn-r-psiEE wYlleHGLKR rIEVVQGFs MippmlSgTe RiATmPLRLa
 251
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 qhFaktiPLR I-ELP1Pp PteAvQWPaL hNsdpaSIWM R--LIQEASr
 301
 HW17b D1
 BR816 D2
 CIAT899 D1
 NZP2213 D3
 8002 D1
 Consensus
 M--p-----

FIG. 8. Multiple alignment of predicted amino acid sequences of *Rhizobium* sp. strain HW17b NodD and its most similar homologs. Sequences were progressively aligned by using the Pileup program (Genetics Computer Group [8]) as implemented under DNASYSYSTEM (39). Blank spaces indicate agreement at that position with the consensus presented below the alignment, while dots represent the absence of a character in that position. Uppercase letters in the consensus indicate a character in agreement among all sequences, lowercase letters show agreement among three or four sequences, and dashes show agreement among less than three sequences. Strain designations (and sequence database accession numbers are as follows: HW17b D1, *Rhizobium* (*Prosopis*) sp. strain HW17b NodD1 (GenBank U16154); BR816 D2, *R. tropici* BR816 NodD2 (GenBank L01272); CIAT899 D1, *R. tropici* CIAT899 NodD1 (GenBank L04660); NZP2213 D3, *R. loti* NZP2213 NodD3 (PIR S21410); and 8002 D1, *R. leguminosarum* bv. *phaseoli* 4292 NodD1 (SwissProt P23718).

EPS-I. We considered the possibility that strain AK631, which effectively nodulates alfalfa (33), is unable to nodulate mesquite because it lacks EPS-I. EPS-I is required for symbiosis in *R. meliloti* strains missing certain exopolysaccharide genes (26, 33). None of the nodule isolates from the library of mesquite rhizobia in *R. meliloti* AK631 contained EPS-I, however, so mesquite nodulation by *R. meliloti* AK631 does not require synthesis of EPS-I.

NodD is a primary regulator of *nod* gene expression and functions in host specificity by modulating the bacterial response to plant signals. *Rhizobium* (*Parasponia*) sp. strain NGR234CS cells carrying just the *nodD* genes of rhizobia or bradyrhizobia did not nodulate mesquite, indicating that *nodD* was either not required or was insufficient for host range extension. The mesquite microsymbiont *nodD* genes probably mediate a response to host signals because the cosmid bradyrhizobial pKFG83, lacking the *nodD* gene, could not restore nodulation of *Rhizobium* sp. strain HW27cCS, while *nodD*-

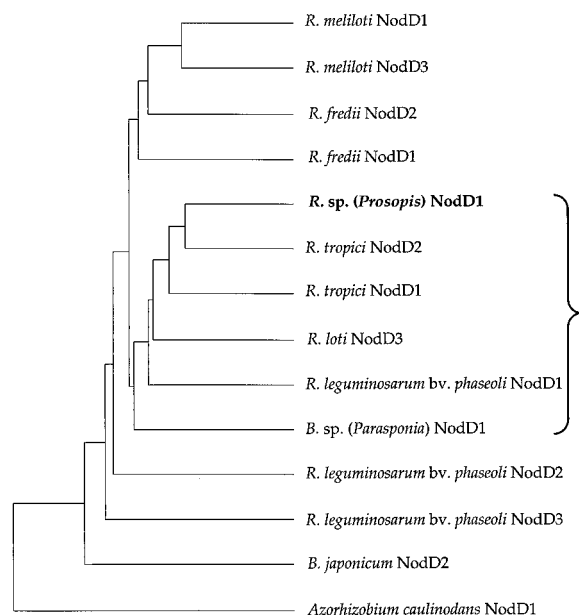


FIG. 9. Dendrogram of distance relationships among representative NodD proteins, including the bracketed branch from strains that infect herbaceous and temperate legumes. The new NodD protein sequence of *Rhizobium (Prosopis)* sp. strain HW17b, which was deduced from the nucleotide sequence shown in Fig. 7, is shown in boldface. The sequences of the NodD proteins used in the alignment represented in this dendrogram have been published and are available from the NCBI sequence database. *R.*, *Rhizobium*; *B.*, *Bradyrhizobium*.

containing clones from either genus could. The broad host range of *Rhizobium (Parasponia)* sp. strain NGR234 (35) is caused in part by the response of the endogenous NodD to flavonoid compounds (14), and the NGR234 NodD could be sufficient to activate *nod* gene transcription in response to mesquite signals. The genetic information from mesquite symbionts that allows *Rhizobium (Parasponia)* sp. strain NGR234CS to nodulate mesquite may produce biochemical modifications of a Nod factor that then elicits a response from mesquite.

Most strains of mesquite rhizobia from surface, intermediate, or deep soil at Harper's Well (41, 45) were shown to have the cloned *nodD* gene from *Rhizobium* sp. strain HW17b on a 6.6-kb *Pst*I fragment, suggesting that we have cloned the *nodD* gene common among wild-type populations of mesquite rhizobia. Strains whose *nodD*-hybridizing fragments were of sizes different from the 6.6-kb fragment had hybridization profiles characterized by changes in the number and distribution of weak bands (Fig. 3). This is suggestive of repetitive sequences associated with *sym* regions of other symbionts (11, 15). Such elements may be cloned with the HW17b *nodD* fragment and may have contributed to the divergent signal profiles seen here.

Rhizobium sp. strain HW27cCS is Nod⁺, and its genome did not hybridize to the *nodD* fragment. Because only one *nodD* gene was isolated from the *Rhizobium* sp. strain HW17b genome in complementation experiments with HW27cCS, it is probable that the *nodD* gene of rhizobia identified in our work is essential for mesquite nodulation in other HW strains of rhizobia. We, therefore, determined the nucleotide sequence of the *Rhizobium* sp. strain HW17b *nodD1* gene.

Sequence comparison studies identified a cluster of five NodD homologs from rhizobia that can infect both herbaceous and tree legumes. Members of these species nodulate herbaceous *Lotus* or *Phaseolus* species and the tree legumes *Mimosa*,

Leucaena, and *Prosopis* species (19, 28, 45). Whether these NodD homologs are all involved in tree nodulation is unknown, but their sequence similarity to the NodD of *Rhizobium* sp. strain HW17b, a symbiont of the tree legume mesquite, suggests this is the case. Understanding the phylogeny of rhizobia is difficult when plasmid-borne determinants are used, but we expect that the relationships identified in this cluster reflect selection during coevolution with available plant hosts.

Amino acid comparisons with the four other NodD proteins showed three regions of conservation (Fig. 8). As expected, one region of strong sequence conservation includes the HTH motif, which is conserved among many bacterial DNA-binding proteins that are involved in transcriptional regulation (17). The C-terminal half of the HTH motif makes up the loop and the recognition helix that in crystallized HTH proteins, complements the shape of the DNA major groove (4), so the region would be expected to vary little among these proteins. Alignment of available sequences from the NodD database indicates that 41% of invariant residues are within the 53 N-terminal positions (data not shown). Positions 1 to 14 of Fig. 8 are fully conserved among the five NodD proteins. The region's proximity to HTH implies its probable significance in functions related to NodD DNA binding. A potential receiver module spans positions 69 to 116 (24). Receiver modules are important in two-component signal transduction schemes, although phosphorylation has not been demonstrated for NodD. The third region includes a part of NodD that when mutated in *Rhizobium leguminosarum*, caused either deficiency of activation of *nod* genes while maintaining autoregulation or led to inducer-independent *nod* gene activation (3). Inferred to interact with inducer molecules, this region includes a stretch strongly similar among NodD proteins and other LysR-type regulators (5, 6). That these features of NodD are conserved in the cluster of five sequences suggests that they play important roles in related functions of the symbiotic regulators.

ACKNOWLEDGMENTS

Several strains or plasmids important to this work were graciously provided by G. Ditta, A. Kondorosi, D. Kuykendall, B. Rolfe, and M. Silverman. We gratefully acknowledge J. Bell, G. Ditta, A. Forsyth, D. Helinski, T. Montanez, V. Newman, D. Smith, D. Tierney, and J. Varnell for useful discussions or help. Help in graphic production or reproduction by R. Goehner and by S. Lamont of the UCSD Microscopy and Imaging Resource is acknowledged and appreciated.

This research was supported by NSF Ecosystems Studies grant BSR-8216814 to R.A.V. and a grant-in-aid from San Diego State University. This work is a contribution to the NSF Jornada Long Term Ecological Research Program under grant no. BSR-8811160.

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